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Epitope analysis of peanut allergen Ara h1 with oligoclonal IgM antibody from human B-lymphoblastoid cells

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Abstract To analyze epitopes of peanut allergen Ara h1, Epstein-Barr virus-transformed human peripheral oligoclonal B-cells were cultured to obtain antibodies to Ara h1. The combined reaction pattern with six oligoclonal antibodies showed there were six antibody binding areas named a to f in Ara h1. We found the novel antibody binding area named "area c" (171–230aa).

Keywords Antibody · Epitope · Epstein-Barr virus · IgM · Peanut allergen

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Introduction

Peanut allergy is one of the most common and serious of the immediate hypersensitivity reactions to foods in terms of persistence and severity of reaction. To date, three major allergens, Ara h1, Ara h2 and Ara h3, and four minor allergens, Ara h4, Ara h5, Ara h6 and Ara h7, have been identified with serum IgE from patients allergic to peanuts (Burks et al. 1992, 1991; Rabjohn et al. 1999; Kleber-Janke et al. 1999). More than 90% of the serum IgE regocnized Ara h1 and Ara h2.

Epstein-Barr virus (EBV) is a herpes virus that infects human B-cells to cause infectious mononucleosis (Counter et al. 1994). It is also known that EBV transforms and immortalizes human B-cells in vitro. Those immortalized cells (B-lymphoblastoid cells, BLCs) secrete antibodies. To analyze the epitopes of peanut allergen, we have obtained human monoclonal antibodies, previously. At first, human peripheral B cells were transformed with EBV, and fused with mouse myeloma cell to form mouse-human hybridomas secreting two monoclonal antibodies to Ara h1. (Shimmoto et al. 2004) However, making human monoclonal antibodies was very time consuming and we have failed to obtain additional monoclonal antibodies.

On the way to preparation of human monoclonal antibodies, we already have many EBV-transformed human BLCs (Shimmoto et al. 1998). While the BLCs stored are oligo-clonal and cells become crisis when cultured for long term (more than 2–6 months),

those BLCs seem to be very useful tool to obtain mixed-oligoclonal antibodies to certain food allergens including Ara h1. In this paper, we showed BLCs secreting oligoclonal IgM antibodies to Ara h1 could recognize known and novel epitopes.

Materials and methods

Preparation of peripheral blood lymphocytes and transformation of the lymphocytes with Epstein-Barr virus

Peripheral blood lymphocytes (PBLs) were prepared from 13 healthy donors by discontinuous density centrifugation on lymphocyte separation medium (GE Healthcare Japan). The PBLs were washed twice with eRDF medium (Kyokuto Seiyaku, Japan) and were then suspended into eRDF medium (10^6 cells/mL) supplemented with 10% fetal calf medium (FCS, Nichirei, Japan), cyclosporin A (2 μg/mL) and 1/10 volume of culture supernatants of marmoset B95-8 cells, containing Epstein-Barr virus (EBV). The cell mixture was then plated into 96-well microculture plates (10^5 cells/well). The medium (eRDF medium supplemented with 10% FCS) was changed every three or four days. After 3 weeks of culture, transformed B cells (B-lymphoblastoid cells, BLCs) and culture supernatants were frozen and stored. The experiment was carried out according to the ethical code for human experiment of National Food Research Institute with informed consent before the first analysis of blood antibodies.

Enzyme-linked immunosorbent assay (ELISA)

Powdered peanut (1.0 g) was extracted with 10 mL of 0.5 mol/L NaCl for 2 h at room temperature. The mixture was centrifuged at 15,000 rpm for 10 min to remove precipitates and floating fatty materials. The supernatant, protein concentration of 12.5 mg/mL, was used as a crude peanuts allergen.

The crude peanut allergen and purified peanut allergen Ara h1 were diluted with 0.05 mol/L $NaHCO_3$ (protein concentration of 1–5 μg/mL), and 0.06 mL of the allergen solution was plated into ELISA plates. The plates were kept at 4 °C for 16 h. The allergen solution was then discarded and the plates were blocked with a blocking reagent (Block Ace, Dainippon Sumitomo Pharma, Japan) for 1 h followed by washing with

Fig. 1 Sequence of synthesized overlapping Ara h1 peptides

1 MRGRVSPLML LLGILVLASV	25 GQATVTVANG NNRKSFNLDE	49 EEEEDEDEEE EGSNREVRRY
2 LLGILVLASV SATHAKSSPY	26 NNRKSFNLDE GHALRIPSGF	50 EGSNREVRRY TARLKEGDVF
3 SATHAKSSPY QKKTENPCAQ	27 GHALRIPSGF ISYILNRHDN	51 TARLKEGDVF IMPAAHPVAI
4 QKKTENPCAQ RCLQSCQQEP	28 ISYILNRHDN QNLRVAKISM	52 IMPAAHPVAI NASSELHLLG
5 RCLQSCQQEP DDLKQKACES	29 QNLRVAKISM PVNTPGQFED	53 NASSELHLLG FGINAENNHR
6 DDLKQKACES RCTKLEYDPR	30 PVNTPGQFED FFPASSRDQS	54 FGINAENNHR IFLAGDKDNV
7 RCTKLEYDPR CVYDPRGHTG	31 FFPASSRDQS SYLQGFSRNT	55 IFLAGDKDNV IDQIEKQAKD
8 CVYDPRGHTG TTNQRSPPGE	32 SYLQGFSRNT LEAAFNAEFN	56 IDQIEKQAKD LAFPGSGEQV
9 TTNQRSPPGE RTRGRQPGDY	33 LEAAFNAEFN EIRRVLLEEN	57 LAFPGSGEQV EKLIKNQKES
10 RTRGRQPGDY DDDRRQPRRE	34 EIRRVLLEEN AGGEQEERGQ	58 EKLIKNQKES HFVSARPQSQ
11 DDDRRQPRRE EGGRWGPAGP	35 AGGEQEERGQ RRWSTRSSEN	59 HFVSARPQSQ SQSPSSPEKE
12 EGGRWGPAGP RERERREEDWR	36 RRWSTRSSEN NEGVIVKVSK	60 SQSPSSPEKE SPEKEDQEEE
13 RERERREEDWR QPREDWRRPS	37 NEGVIVKVSK EHVEELTKHA	61 SPEKEDQEEE NQGGKGPLLS
14 QPREDWRRPS HQQPRKIRPE	38 EHVEELTKHA KSVSKKGSEE	62 NQGGKGPLLS ILKAFN
15 HQQPRKIRPE GREGEQEWGT	39 KSVSKKGSEE EGDITNPINL	63 ILKAFN
16 GREGEQEWGT PGSHVREETS	40 EGDITNPINL REGEPDLSNN	
17 PGSHVREETS RNNPFYFPSR	41 REGEPDLSNN FGKLFEVKPD	
18 RNNPFYFPSR RFSTRYGNQN	42 FGKLFEVKPD KKNPQLQDLD	
19 RFSTRYGNQN GRIRVLQRFD	43 KKNPQLQDLD MMLTCVEIKE	
20 GRIRVLQRFD QRSRQFQNLQ	44 MMLTCVEIKE GALMLPHFNS	
21 QRSRQFQNLQ NHRIVQIEAK	45 GALMLPHFNS KAMVIVVVNK	
22 NHRIVQIEAK PNTLVLPKHA	46 KAMVIVVVNK GTGNLELVAV	
23 PNTLVLPKHA DADNILVIQQ	47 GTGNLELVAV RKEQQQRGRR	
24 DADNILVIQQ GQATVTVANG	48 RKEQQQRGRR EEEEDEDEEE	

Table 1 Detection of anti peanut antibodies in supernatant of human BLCs Human peripheral B-cells were transformed by EBV infection (see Section "Materials and methods") and the number of oligoclonal cells secreting antibodies specific to peanut allergen was detected by ELISA

Allergen	Class of antibody reacted			
	IgM	IgG	IgA	IgE
Crude extract	80	5	0	0
Ara h1	26	2	3	0

*The number of transformed cells analyzed was 5,142

phosphate-buffered saline containing 0.05% Tween-20 (PBS-T). The culture supernatants of BLCs (0.05 mL) were pipetted into the plates and incubated for 1 h at room temperature, the plates were washed again, and then 0.05 mL of antibodiy to human immunoglobulins conjugated with horseradish peroxidase (Biosource International, USA) was added to the plates. After 1 h of incubation at room temperature, the plates were washed six times and peroxidase activity immobilized on the plates was measured by adding 0.1 mL of substrate solution (0.3 mg/mL 2,2′-azino-di-[3-ethyl benzthiazolin sulfonic acid] ABTS, 0.03% H_2O_2 in 0.1 mol/L citrate buffer pH 4.0) and the absorbancy of reaction products was measured at 415 nm.

Analysis of epitopes of peanut allergen Ara h1 by the multi-pin overlapping peptide method

We synthesized the series of 20-amino acid-overlapping peptide based on amino acid sequence of Ara h1 protein (Fig. 1). The peptides were synthesized on the multi-pin apparatus (Mimotope, Australia) by solid-phase synthesis method with immobilized C-terminus and free NH_2. Supernatants of BLCs were pipetted into a 96-well plate, and multi-pin peptide were placed on the plate and reacted for 1 h at room temperature. The multi-pin were washed with PBS-T and reacted with anti-human IgM antibody conjugated with horseradish peroxidase. After washing, the enzyme activity on the pins was measured in a 96-well plate.

Result and discussion

We obtained more than 5,000 transformed human BLCs which were derived from thirteen donors. The supernatants of the BLCs were analyzed by ELISA for antibodies against the crude peanut allergen, and the major peanut allergen Ara h1. As shown in Table 1, we obtained IgM, IgG, and IgA antibody-secreting BLCs against the Ara h1. During the culture of those BLCs for 1–2 months, some BLCs stopped secreting antibodies, and we finally chose six oligoclonal BLC-cultures secreting oligoclonal IgM class antibodies stably (BLC#A-117, B-221, C-272, D-181, E-39, and F-61).

Each oligoclonal antibody reacted with two to four regions of the series of synthesized peptides and Fig. 2 combined the results of the six oligoclonal antibodies. The combined reaction pattern showed there were six antibody binding areas named "a"

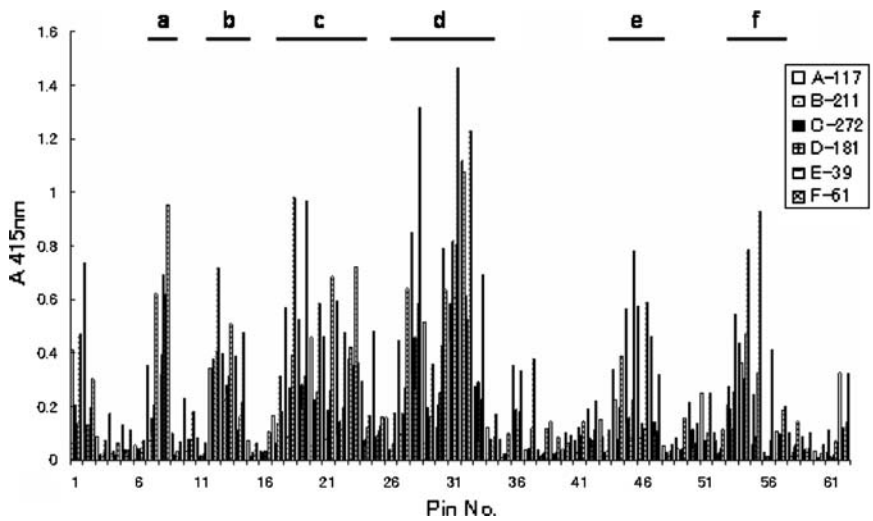

Fig. 2 Epitope analysis of Ara h1 with human BLCs. Synthesized overlapping peptides based on amino acid sequence of Ara h1 peanut allergen reacted with oligoclonal IgM from human BLCs (A-117, B-211, C-272, D-181, E-39 and F-61)

Fig. 3 Amino acid sequence of Ara h1. Hatched area named a, b, c, d. e, and f were region of epitopes estimated multi-pin peptides analysis (this study), and underlined areas were previously shown areas with serum IgE from patients of peanut allergy. a:71–90, b:111–140, c:171–230, d:271–330, e:441–460, f:531–550 (amino acid)

(71–90aa) to "f" (531–550aa) in the Fig. 2. Figure 3 shows the amino acid sequence of Ara h1. The epitopes detected with IgE antibodies from patients allergic to peanut (Burks et al. 1995) were underlined, areas detected with the oligoclonal IgM antibodies in this study were hatched. While our oligoclonal IgM antibodies could not detect some epitopes, we found the novel common antibody binding area named area "c". Further detailed epitope analysis of area "c" would be needed to clarify the reason for the absence of IgE binding epitope in the area "c".

In this study, we showed the human oligoclonal IgM antibodies secreted by EBV-transformed BLCs were useful tool for analysis of food allergens. Long term culture of those BLCs seemed very difficult, because we have lost several antibodies detected at Table 1. So, it seems to be important to obtain supernatants from BLCs secreting certain epitope of food allergens within one to 2 months before BLCs stop secreting antibodies.

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